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An extended mini-complement factor H molecule ameliorates experimental C3 glomerulopathy

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Abnormal regulation of the complement alternative pathway is associated with C3 glomerulopathy. Complement factor H is the main plasma regulator of the alternative pathway and consists of 20 short consensus repeat (SCR) domains. Although recombinant full-length factor H represents a logical treatment for C3 glomerulopathy, its production has proved challenging. We and others have designed recombinant mini-factor H proteins in which 'non-essential' SCR domains have been removed. Here, we report the *in vitro* and *in vivo* effects of a mini-complement factor H protein, $FH^{1-5\wedge18-20}$, using the unique factor H–deficient (*Cfh* − / −) mouse model of C3 glomerulopathy. $FH^{1-5\wedge18-20}$ is comprised of the key complement regulatory domains (SCRs 1–5) linked to the surface recognition domains (SCRs 18–20). Intraperitoneal injection of $FH^{1-5\wedge18-20}$ in *Cfh* − / − mice reduced abnormal glomerular C3 deposition, similar to full-length factor H. Systemic effects on plasma alternative pathway control were comparatively modest, in association with a short half-life. Thus, $FH^{1-5\wedge18-20}$ is a potential therapeutic agent for C3 glomerulopathy and other renal conditions with alternative pathway-mediated tissue injury.

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Factor H (FH, 150 kDa) is the key regulator of complement activation in plasma.[1,2] FH inhibits the C3b amplification loop by preventing formation and promoting dissociation of the alternative pathway (AP) C3 convertase, and by acting as a cofactor for factor I (FI)–mediated C3b cleavage.[1,3] It comprises 20 short consensus repeat (SCR) domains, with amino (N)-terminal SCRs 1–4 accounting for its AP regulatory functions. FH is targeted to specific tissues via two binding domains that recognize different heparan sulfate species.[4] The principle binding site for heparan sulfate species of the retina is located in SCR 7, whereas SCRs 19–20 target glomerular heparan sulfate.[5] Indeed, C-terminal SCRs 19–20 are critical for interaction with tissue-bound C3b and cell surface polyanions, enabling FH to regulate the AP on host cell surfaces.[2,6] Uncontrolled C3 activation via the AP because of deficiency or dysfunction of FH is associated with the development of C3 glomerulopathy (C3G).[7] The pathological hallmark of C3G is predominant C3 accumulation within the glomerulus, leading to end-stage kidney disease within 10 years of diagnosis in ~40% of patients.[8] Currently, there is no proven treatment for C3G, although a small prospective trial and several case reports suggest that the C5 inhibitor eculizumab may be beneficial in some patients.[9–11]

Mice with targeted homozygous deficiency of murine FH (*Cfh* − / −) demonstrate low plasma C3 levels and a linear pattern of C3 staining in the glomerulus, and eventually develop membranoproliferative glomerulonephritis.[12] Murine experimental C3G thus provides a useful model for studying the pathogenetic mechanisms and response to novel therapeutics of C3G. Injection of human FH or murine Cfh has been shown to restore plasma C3 levels and reduce glomerular C3 deposition in *Cfh* − / − mice at 24 h.[13,14] Accordingly, recombinant FH has been proposed as a logical treatment for establishing physiological AP control and halting disease progression in patients with C3G. However, because of the size and complexity of the FH protein, production of therapeutic quantities represents a significant challenge.[15–17] Here, we describe the generation and successful administration in *Cfh* − / − mice of a mini-FH protein ($FH^{1-5\wedge18-20}$), which is comprised of the key functional domains of FH. $FH^{1-5\wedge18-20}$ reduced glomerular C3 reactivity similar to full-length FH while also partially restoring plasma

C3 levels. These data indicate a plausible therapeutic role for $FH^{1-5\wedge18-20}$ in patients with C3G and other disorders of complement regulation.

RESULTS

Generation of FH^{1-5} and the Newcastle mini-FH ($FH^{1-5\wedge18-20}$) proteins

For our mini-FH construct, we included the first five SCRs of the N-terminal region of FH, as we and others have found that SCRs 1–5 of FH has improved function over SCRs 1–4 (Supplementary Figure S1 online; Huang *et al.*,[18] Cheng *et al.*,[19] and Gordon *et al.*[20]); SCR 5 is also the target of monoclonal antibody OX-24,[21] allowing an additional means to purify/identify the construct. We included SCR 18 at the C terminus to provide spacing to the construct and included a hexa-histidine tag as the primary means of purification, as well as providing additional flexibility/spacing between SCRs 5 and 18. $FH^{1-5\wedge18-20}$ and FH^{1-5} were expressed in Chinese hamster ovary cells and yielded 2 and 4 mg/l, respectively. After two-step purification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis confirmed the purity of both FH^{1-5} and $FH^{1-5\wedge18-20}$, and that they migrated at their predicted molecular weight of ~36 and 59 kDa, respectively (Figure 1a). To assess the binding of $FH^{1-5\wedge18-20}$ to key ligands, microtiter plates were coated with C3b or heparin (a model polyanion) and binding of equimolar amounts of FH, FH^{1-5}, and $FH^{1-5\wedge18-20}$ measured. All three FH proteins bound to C3b, whereas only FH and $FH^{1-5\wedge18-20}$ bound heparin as detected by a polyclonal anti-FH (Figure 1b and c). FH and $FH^{1-5\wedge18-20}$ bound to C3b or heparin in an equivalent manner and could be inhibited by specific monoclonal antibody, confirming the specificity of the interaction in this assay. In light of recent findings regarding deregulation of FH function by the FHR proteins[22] and to establish if $FH^{1-5\wedge18-20}$ was also susceptible to deregulation, we carried out a C3b-binding/competition assay using equimolar amounts of FH, FH^{1-5}, and $FH^{1-5\wedge18-20}$ and increasing doses of recombinant FHR1 or FHR5. In this assay, $FH^{1-5\wedge18-20}$ was significantly more resistant to deregulation compared with FH at all concentrations of FHR (Figure 1d and e) but was still deregulated at the highest FHR doses (1.8 and 0.6μmol/l, respectively).

Fluid-phase regulatory capacity of recombinant $FH^{1-5\wedge18-20}$ is comparable to serum-derived FH

FI requires a cofactor such as FH to mediate cleavage of the C3b α-chain. The ability of $FH^{1-5\wedge18-20}$ to serve as a cofactor for FI was initially tested in a standard fluid-phase cofactor assay[23] and then in an SRBC (sheep red blood cells) assay.[24] C3b, FI, and a cofactor (FH, FH^{1-5}, or $FH^{1-5\wedge18-20}$) were incubated at varying equimolar concentrations. Encouragingly, $FH^{1-5\wedge18-20}$ performed significantly better than FH^{1-5} and was consistently more potent than serum-derived FH in the protection of C3b-coated SRBCs from lysis in this assay (Figure 2a). However, while comparable to FH, $FH^{1-5\wedge18-20}$ was not superior in the classical fluid-phase assays (Supplementary Figure S2 online). We also measured the decay-accelerating activity of FH and the recombinant constructs using C3b-coated SRBCs; again, FH and $FH^{1-5\wedge18-20}$ demonstrated 10-fold greater ability to decay the C3b convertase on SRBCs compared with FH^{1-5}. However, in this assay $FH^{1-5\wedge18-20}$ decay-accelerating activity function was indistinguishable from that of FH (Figure 2b). Overall, these data suggest that $FH^{1-5\wedge18-20}$ has maintained the complement regulatory function of FH.

$FH^{1-5\wedge18-20}$ is highly efficient in protecting SRBCs from complement attack by aHUS patient or FH functionally depleted serum

SRBCs are normally resistant to AP-driven complement attack because FH readily binds to their surface via its C terminus. This fact has been exploited by Sanchez-Corral *et al.*[25] who demonstrated that serum from patients with mutations in FH that affected the C terminus would readily lyse SRBCs. We have previously used this assay to confirm a defect in complement regulation in a patient with an FH hybrid gene.[26] Addition of FH or $FH^{1-5\wedge18-20}$ to serum from an aHUS (atypical hemolytic uremic syndrome) patient with C-terminus mutation was highly effective in blocking SRBC lysis (Figure 3b), confirming that increasing FH levels is sufficient to reverse an inherent regulatory defect in patient serum, with $FH^{1-5\wedge18-20}$ being equivalent to an equimolar concentration of FH. Using OX-24 to block FH function in the normal serum, which prevents FH binding to C3b on the erythrocyte surface,[27] we were able to show that functional loss of FH regulatory function by antibody blockade of the N terminus of FH can also be reversed by the addition of FH or $FH^{1-5\wedge18-20}$ (Figure 3c). The data indicate, in these cell-based assays, that $FH^{1-5\wedge18-20}$ is marginally more effective compared with FH in complement regulatory function in patient serum and may be useful for treatment of patients with either defective FH or antibodies that block FH function.

$FH^{1-5\wedge18-20}$ reduces glomerular C3 staining in *Cfh* −/− mice at doses that incompletely restore plasma C3 levels

We assessed the ability of $FH^{1-5\wedge18-20}$ to influence plasma and glomerular C3 *in vivo* by administering the protein to *Cfh* −/− mice (Figure 4a). After a single intraperitoneal 12 nmol dose of either $FH^{1-5\wedge18-20}$ or FH^{1-5}, plasma C3 levels significantly increased at 2 and 6 h after injection reaching ~20% of wild-type levels at 6 h (Figure 4a). Both injected proteins were detectable in plasma at 2 h after injection but absent or barely detectable at the 6 h time point (Figure 4b). In contrast, after a single injection of 3 nmol of serum-derived FH (the dose used successfully by Fakhouri *et al.*[14]), the injected FH was detectable at comparable concentrations 2 and 6 h after injection, and plasma C3 increased to ~60% of wild-type levels at the 6 h time point (Figure 4a and b). Glomerular C3 immunostaining fluorescent intensity was significantly reduced 6 h after injection of $FH^{1-5\wedge18-20}$ to a comparable degree to that seen following FH (Figure 4c). No significant reduction in glomerular C3 staining was seen in

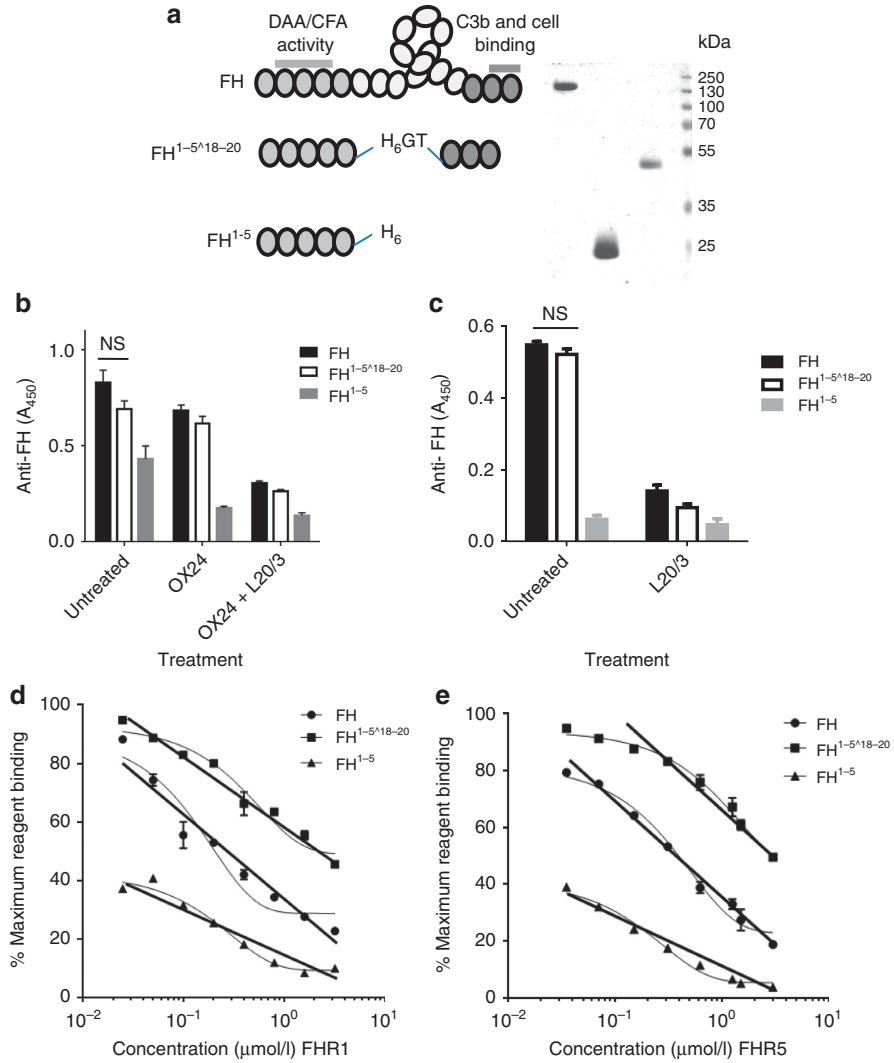

Figure 1 | Initial analysis of recombinant FH$^{1-5}$ and FH$^{1-5\hat{}18-20}$ protein. (**a**) Serum-purified full-length factor H (FH) or Chinese hamster ovary (CHO) cells expressed and purified recombinant FH$^{1-5}$ and FH$^{1-5\hat{}18-20}$ were visualized on 12.5% sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie staining. Proteins are represented schematically next to bands and molecular weight (MW) markers are indicated. Basic functional properties of the new construct were determined in enzyme-linked immunosorbent assay (ELISA) assays. To detect binding of FH$^{1-5}$ and FH$^{1-5\hat{}18-20}$ to (**b**) C3b and (**c**) heparin, FH proteins (10 nmol/l) were applied to appropriately coated ELISA plates. Preincubation of samples with monoclonal antibodies OX-24 and L20/3 or L20/3 (at 1.5 molar equivalence to FH) was carried out as indicated. Serum-purified FH acted as a positive control. No significant change was noted between FH$^{1-5\hat{}18-20}$ and FH binding to C3b or heparin using two-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test. Binding of FH proteins was detected using a goat anti-human FH polyserum, followed by a horse radish peroxidase (HRP)–conjugated donkey anti-goat Ig secondary antibody. The ability of (**d**) rFHR1 and (**e**) rFHR5 protein to interfere with FH$^{1-5}$ and FH$^{1-5\hat{}18-20}$ binding to a heavily C3b-coated surface is shown. Data shown are a composite of three similar experiments, mean ± s.e.m. is shown. Two-way ANOVA with Bonferroni multiple comparisons test confirms that FH$^{1-5}$ and FH$^{1-5\hat{}18-20}$ bind significantly worse or significantly better, respectively, compared with FH to C3b in the presence of FHRs. CFA, cofactor activity; DAA, decay-accelerating activity; NS, nonsignificant.

the group that received a single injection of the FH$^{1-5}$ protein (Figure 4c). We were able to detect both FH and FH$^{1-5\hat{}18-20}$, but not FH$^{1-5}$ protein, in the glomerulus using the OX-24 antibody (Figure 4d). We next injected FH$^{1-5\hat{}18-20}$ at 6-hourly intervals over a 24 h period at which time point we assessed glomerular C3 staining. Similar to our findings 6 h after injection of the FH$^{1-5\hat{}18-20}$ protein, glomerular C3 staining intensity was significantly reduced 24 h following multiple injections, but in these animals we also noted areas of mesangial staining, comparable to that seen previously 24 h following injection of full-length FH (Fakhouri *et al.*[14]; Supplementary Figure S3A and B online). No change in glomerular C3d reactivity was detectable (Supplementary Figure S3B).

DISCUSSION

Given the robust association of FH deficiency/dysfunction with severe, progressive renal disease, therapeutic evaluation of full-length recombinant FH has long seemed merited. However, this has not been possible. FH is a large

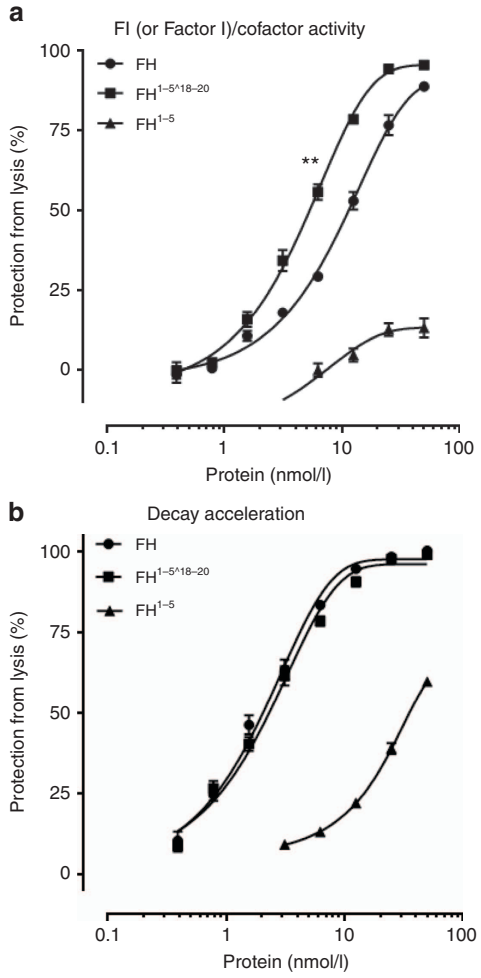

Figure 2 | $FH^{1–5^{18–20}}$ cell membrane complement regulatory activity comparable to factor H (FH). (**a**) FI (or Factor I) cofactor activity: C3b-coated sheep erythrocytes were exposed to 2.5 µg/ml factor I (FI) and either FH (0.8–100 nmol/l), $FH^{1–5^{18–20}}$ (0.8–100 nmol/l), or $FH^{1–5}$ (6–750 nmol/l) and incubated at 25 °C inactivate the C3b. Remaining C3b was induced to create C3 convertase whose level was then determined by the amount of lysis of red cells after incubation with FB- and FH-depleted serum in phosphate-buffered saline (PBS)/20 mmol/l ethylenediaminetetraacetic acid (EDTA). (**b**) Decay-accelerating function of $FH^{1–5^{18–20}}$ was assayed using sheep erythrocytes precoated with AP convertase (see Materials and Methods). Data shown are mean ± s.e.m. of at least six independent titrations and a composite of two independent experiments (cofactor activity (CFA)) and four independent experiments (decay-accelerating activity). $FH^{1–5^{18–20}}$ has significantly improved CFA function compared with FH using two-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test, $^{**}P > 0.001$.

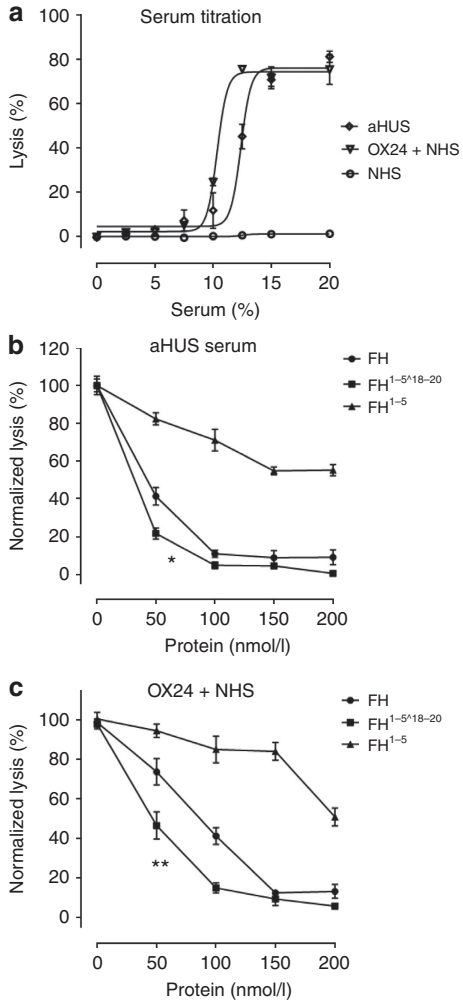

Figure 3 | $FH^{1–5^{18–20}}$ protects sheep red blood cells (SRBCs) from lysis by atypical hemolytic uremic syndrome (aHUS) patient sera. (**a**) Serum from an aHUS patient known to carry a heterozygous CFH/CFHR1 (complement factor H/CFHR1) hybrid gene lyses SRBCs in a dose-dependent manner, whereas serum from a normal individual does not. Increasing the quantity of OX-24 monoclonal antibody (mAb) added to 20 µl normal serum blocks factor H (FH) function in a dose-dependent manner. Volume of serum or OX-24 Ab added is indicated on the X axis. (**b**) aHUS patient serum (12.5 µl) was spiked with increasing concentration of FH reagents as indicated and prevented lysis of red cells. (**c**) Normal patient serum was mixed with OX-24 (15 µg/20 µl serum) before the addition of increasing concentration of FH/reagent as indicated. SRBC lysis with serum but no FH reagent was adjusted to represent 100% lysis. Data shown are mean ± s.e.m. of six independent titrations and a composite of two independent experiments. $FH^{1–5^{18–20}}$ has significantly improved function compared with FH by two-way analysis of variance (ANOVA), with Bonferroni multiple comparisons test. $^{*}P > 0.05$ and $^{**}P > 0.001$.

glycoprotein with eight confirmed N-glycosylation sites and 40 disulfide bonds,[28] making large-scale manufacture technically difficult. Even if production issues can be addressed,[29] recombinant FH will lack the normal mammalian glycosylation pattern, predisposing it to rapid clearance by natural antibody/innate immune recognition.[30] By contrast, production of recombinant mini-FH proteins is technically feasible and has the added theoretical advantage of circumventing immune evasion by strains of *Neisseria meningitides*.[31] The latter are able to hijack FH by binding of SCR 6 through the meningococcal cell surface protein, FH-binding protein.[32,33] Here, we show for the first time that a mini-FH protein, $FH^{1–5^{18–20}}$, is able to regulate the AP *in vivo*, effectively reversing glomerular C3 accumulation and transiently increasing plasma C3 levels in *Cfh* − / − mice.

Administered as a single 12 nmol injection, $FH^{1–5^{18–20}}$ had a significant effect on plasma C3 levels between 2 and 6 h, and

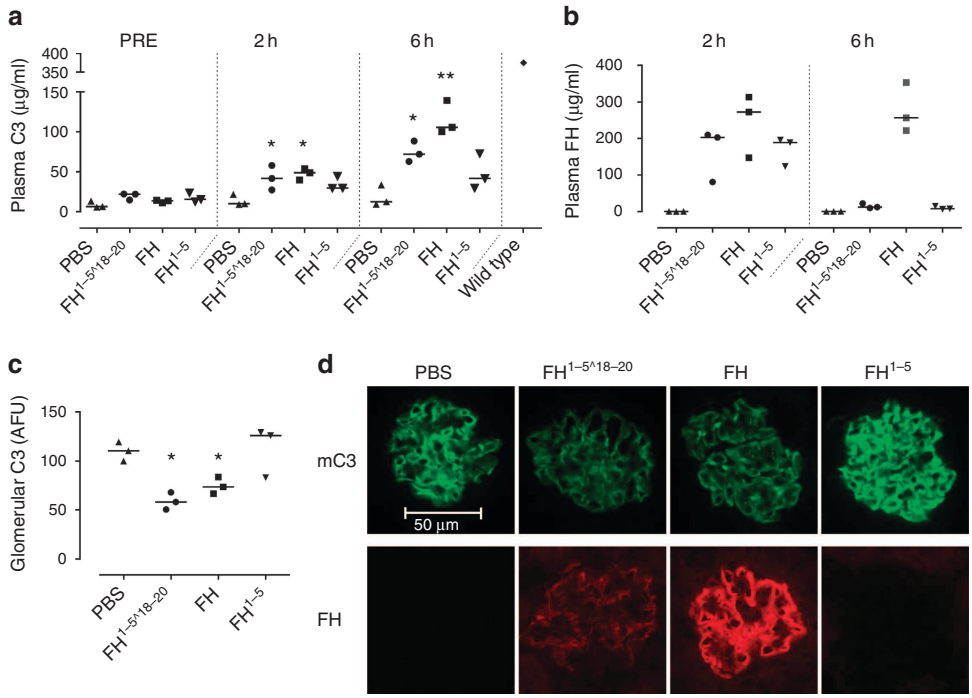

Figure 4 | Effect of FH$^{1–5\text{^}18–20}$ on plasma C3 levels and glomerular C3 staining in *Cfh* –/– mice. (**a**) Plasma C3 levels increased significantly 2 and 6 h after injection of 3 nmol full-length factor H (FH) or 12 nmol FH$^{1–5\text{^}18–20}$ compared with phosphate-buffered saline (PBS). (**b**) FH (3 nmol) was detected at comparable levels 2 and 6 h after administration, whereas FH$^{1–5\text{^}18–20}$ (12 nmol) and FH$^{1–5}$ (12 nmol) had fallen markedly after the 2 h time point. (**c**) Glomerular C3 intensity was significantly reduced 6 h after either 3 nmol FH or 12 nmol FH$^{1–5\text{^}18–20}$ compared with PBS. (**d**) Representative images of (upper panel) glomerular C3 and (lower panel) injected proteins. Illustrative scale bar shown is applicable to all images. *$P<0.05$, **$P<0.01$ vs. PBS, one-way analysis of variance (ANOVA) with Bonferroni multiple comparison test. Horizontal bars denote the median value.

produced an equivalent reduction in glomerular C3 reactivity to full-length FH (Figure 4a). As expected because of the short time (6 h) after infusion of FH or FH$^{1–5\text{^}18–20}$, no evidence of glomerular immunoglobulin G (IgG) deposition was detected (data not shown). A novel finding is that this effect on glomerular C3 staining was associated with detection of administered FH or FH$^{1–5\text{^}18–20}$ in a linear staining pattern within the glomerulus. This could indicate the therapeutic targeting of FH$^{1–5\text{^}18–20}$ to the site of pathological C3 turnover within the kidney, which would be as expected as SCRs 19–20 are known to target glomerular heparan sulfate.[5] Thus, in kidney pathologies that are mediated through deficiency or functional deficits of FH, supplementation with FH$^{1–5\text{^}18–20}$ is expected to be therapeutic. By contrast, FH$^{1–5\text{^}18–20}$ would be less beneficial in retinal pathology (because of the absence of targeting provided in SCRs 6–8; Langford-Smith *et al.*[4] and Clark *et al.*[5]), although this remains to be formally tested. An equivalent total 12 nmol dose, delivered in 6-hourly 3 nmol increments, also reduced glomerular C3 reactivity at the 24 h end point (Supplementary Figure S3 online). The results demonstrate that FH$^{1–5\text{^}18–20}$, unlike FH$^{1–5}$, engages the same therapeutic mechanism as FH and is thus suited for replacement therapy. However, in the absence of detailed *in vivo* dose–response experiments, the present study does not allow a definitive conclusion on the relative efficacies of FH$^{1–5}$, FH$^{1–5\text{^}18–20}$, and FH. *In vitro*, FH$^{1–5\text{^}18–20}$ demonstrated comparable or improved function to that of FH in the red cell-based assays (Figures 2 and 3). An ability to protect cells from complement attack by aHUS patient sera or serum spiked with FH function-blocking antibody (Figure 3b and c) by FH$^{1–5\text{^}18–20}$ suggests the therapeutic potential in the clinical setting of aHUS and in patients who present with anti-FH autoantibodies. However, the successful treatment of autoantibody positive patients with FH$^{1–5\text{^}18–20}$ may depend on the amount of 'free' autoantibody and the target site of the autoantibody. Encouragingly, recent data suggests that anti-FH autoantibodies associated with C3G are of lower affinity, don't readily form immune complexes and don't generally disturb the interaction of FH with C3b,[34] suggesting FH$^{1–5\text{^}18–20}$ could be effective in C3G where anti-FH autoantibodies are present. Furthermore, the ability of FH$^{1–5\text{^}18–20}$ to resist deregulation by FHR1 and FHR5 may also be an advantage in subtypes of C3G where the putative pathogenic mechanism is FH deregulation by abnormal FHR proteins, although we have not directly tested that herein. By contrast, the fluid-phase (Supplementary Figure S2 online) and enzyme-linked immunosorbent assay (ELISA)-based assays (Figure 1b) used suggest a potential reduced efficacy of FH$^{1–5\text{^}18–20}$ to interact with C3b compared with FH under certain circumstances. Ongoing studies comparing our mini-FH construct to the previously reported versions[35,36] (and TT30, that is, CR2 SCRs 1 to 4–FH SCRs 1 to 5), in dose–response studies on human erythrocytes, aim to

address these apparent disparities; these are likely because of subtle (or inherent) differences in targeting preferences of these constructs, giving each construct an advantage for a particular surface.

$FH^{1-5\wedge18-20}$ is the largest of the mini-FH constructs generated to date,[35,36] comprising SCRs 1–5, a short internal hexa-histidine tag linker, and SCRs 18–20. We also chose to use mammalian expression systems to minimize any glycosylation differences that may arise in the production of $FH^{1-5\wedge18-20}$ and reduce potential problems associated with immune clearance that may limit the use of the Pichia-[29,36] or Baculovirus-[35] produced FH recombinant proteins.[30] It was therefore surprising that serum half-life was apparently so short (Figure 4b), although that said, it is similar to that of TT30, which is rapidly cleared from the circulation in the *Cfh*−/− model (conference report/abstract; Ruseva *et al.*[37]) and cleared in ~9 h after intravenous injection into BALB/c mice.[18] Both constructs would be considered as large enough to avoid renal filtration[38] and we could not detect $FH^{1-5\wedge18-20}$ in the urine of wild-type mice injected with a 6 nmol dose at any time point during 24 h analysis (background signal, below limit of detection, data not shown). The clearance process appears to be independent of the presence of glomerular C3 as $FH^{1-5\wedge18-20}$ was also rapidly lost from the serum of injected wild-type C57BL/6 mice (Supplementary Figure S4 online). In contrast, FH has a serum half-life of ~2 days when administered to *Cfh*−/− mice.[14] Therefore, removal of the SCRs 6–17 appears to have reduced the expected serum half-life by up to 50-fold. Detection of FH and $FH^{1-5\wedge18-20}$ within the glomeruli was only visualized using a high dose of biotinylated OX-24 antibody, which may reflect low levels or reduced accessibility to the antigenic site of the injected proteins, highlighting the need for detailed pharmacokinetic analysis using directly labeled reagents. Furthermore, our *in vivo* data addresses the effectiveness of $FH^{1-5\wedge18-20}$ in the absence of endogenous FH, but the efficacy of supplementation of normal levels of FH with $FH^{1-5\wedge18-20}$ remains to be seen. Data from experiments in *Cr2*−/− mice, which demonstrate higher complement turnover in the presence of normal FH levels,[39] suggest that increasing levels of $FH^{1-5\wedge18-20}$ can prevent increased complement turnover (and its downstream effects; manuscript in preparation), thereby providing some evidence that FH functional supplementation is feasible.

In conclusion, short-term efficacy of $FH^{1-5\wedge18-20}$ in murine experimental C3G offers the exciting prospect of an effective treatment for C3G and other complement-mediated renal pathologies. Despite the short serum half-life of $FH^{1-5\wedge18-20}$, we believe these *in vivo* data strengthen the case for further development and preclinical evaluation of mini-FH therapeutics.

MATERIALS AND METHODS

Proteins and antibodies

FH, C3b, FI, factor B (FB), factor D (FD), and the goat anti-human FH polyserum were purchased from Complement Technologies (Tyler, TX). FH used in the *in vivo* studies was purified from normal human serum, in-house, using an OX-24 column. Horse radish peroxidase (HRP)–conjugated bovine (805-035-180-JIR) or donkey (705-036-147-JIR) anti-goat immunoglobulin polyserum or sheep anti-mouse immunoglobulin G (515-035-071-JIR) was purchased from Stratech Scientific (Peterborough, UK). Human C3 for use in the C3 convertase assay was purified from human serum using polyethylene glycol precipitation, anion, and gel filtration chromatography as described previously.[40] The L20/3 and C18/3 anti-human FH antibodies were purchased from BioLegend (London, UK). The FH monoclonal antibody OX-24 was purified from hybridoma (catalog no. 00010402; Sigma, Dorset, UK) tissue culture supernatant, in-house, by protein G chromatography. Biotinylation or attachment of OX-24 to normal human serum–activated HiTrap columns was carried out in strict accordance with the manufacturer's guidance (Lightening link, Innova Biosciences, Cambridge, UK and GE Healthcare, Buckinghamshire, UK, respectively).

Mice

Cfh−/− mice were generated as described previously[12] and backcrossed onto the C57BL/6 genetic background. Mice used were 9–10 weeks old and sex matched. Animals were housed in the animal facility at Imperial College, London, UK, and all experiments were conducted in accordance with institutional guidelines and approved by the UK Home Office. Before each experiment, all mice tested negative for dipstick hematuria (0) and proteinuria (⩽1+) using Hema-Combistix (Siemens, Berlin, Germany).

Construct design

The recombinant FH SCRs 1–5 expression vector was generated by PCR amplification of SCRs 1–5 (residues 1–323) from an in-house cloned and verified pBluescript vector containing the complete FH cDNA (NP_000177.2) using primers to introduce a *Sal*I restriction site at the 3′ end and a hexa-histidine tag, stop codon, and *Nhe*I restriction site at the 5′ end (fwd, 5′-ATAGCTGTCGACGCCACCATGAGACTTCTAGCAA-3′ and rev, 5′-CGCGCTAGCTTAATGATGATGATGATGATGTTTCAAGGTACATCTC-3′, respectively). For recombinant $FH^{1-5\wedge18-20}$, SCRs 1–5 was amplified using the forward primer above and a new reverse primer to introduce a hexa-histidine tag and *Kpn*I restriction site (5′-GAGATGTACCTTGAAACATCATCATCATCATCATGGTACCATCGACTG-3′). SCRs 18–20 were then amplified with primers introducing a *Kpn*I restriction site at the 3′ end and a stop codon and *Nhe*I restriction site at the 5′ end (5′-ACATATGGTACCACCTCCTGTGTGAATTCC-3′ and 5′-TGTTATGCTAGCTTATCTTTTTGCACAAGTTGG-3′, respectively). *CFHR1* (NM-002113) and *CFHR5* (NM-030787) were PCR cloned from commercially available plasmids (IMAGE Consortium; Source Bioscience, Nottingham, UK) with flanking *Xba*I/6× His+*Bam*HI or *Spe*I/6× His+*Bam*HI sequences, respectively. All constructs were cloned into the pDR2EF1α mammalian expression vector[41,42] using the appropriate restriction sites, sequence verified, and transfected into Chinese hamster ovary cells using jetPEI (Polyplus; VWR, Leicestershire, UK) following the standard protocols. The proteins were batch purified using nickel-affinity chromatography according to the manufacturer's guidance (GE Healthcare). $FH^{1-5\wedge18-20}$ was polished using an in-house-generated OX-24 affinity column. Extinction coefficients (ε) and theoretical molecular weights of recombinant (r)FHR1 (36.5 kDa, ε 65,080), rFHR5 (63.3kDa, ε 94,500), FH^{1-5} (35.5 kDa, ε 59,580), and $FH^{1-5\wedge18-20}$ (58.9 kDa, ε 98,720) were calculated using the ExPASy Protparam tool (Swiss Institute of Bioinformatics, Lausanne, Switzerland).

Ligand binding ELISAs

For C3b binding, C3b (5 μg/ml in phosphate-buffered saline (PBS)) was coated to Nunc Maxisorp (Dutscher-Scientific, Grays, UK) 96-well plates overnight at 4 °C. Nonspecific binding was blocked with PBS/2% bovine serum albumin (BSA) for 2 h at room temperature. FH, FH^{1-5}, and $FH^{1-5\wedge18-20}$ were added at 10 nmol/l in PBS/2% BSA, and incubated for 2 h at room temperature. Preincubation of samples with monoclonal antibodies OX-24 and L20/3 or L20/3 at 1.5 molar equivalent to FH was carried out as indicated. Goat anti-human FH polyserum, followed by HRP–conjugated donkey anti-goat immunoglobulin polyserum, was used for detection of the FH proteins. TMB (3,3′,5,5′-tetramethylbenzidine) substrate was used to develop the assay, after stopping with 10% H_2SO_4, and the absorbance was read at 450 nm.

For heparin binding, the plates were coated with poly-L-lysine (molecular weight 30–150 kDa; Sigma) at 50 μg/ml in H_2O overnight at 4 °C. After washing with PBS/0.05% Tween-20, heparin (Sigma) was added at 25 μg/ml in H_2O and then incubated for 2 h at room temperature. After washing and blocking, the protocol was followed as for C3b binding.

For the FHR C3b binding and competition ELISA, as per Goicoechea de Jorge *et al.*,[22] 25 μg/ml of C3b was coated to Nunc Maxisorp plates in a carbonate buffer with pH 9.6. Plates were washed in PBS/0.01% Tween and incubated with PBS/2% BSA for 2 h. Equimolar concentration of FH, FH^{1-5}, or $FH^{1-5\wedge18-20}$ were mixed 1:1 with decreasing concentration of FHR1 or FHR5 protein starting from 1.8 and 0.6 μmol/l, respectively, and applied to the plate. After 2 h at room temperature, plates were washed 4× and then incubated with a 2 μg/ml OX-24. After 1 h, plates were washed again and incubated with a 1/1000 dilution of sheep anti-mouse immunoglobulinG-HRP. Plates were washed 4× and developed as above.

SRBC–based hemolytic assays

(a) The FH loss-of-function assay was undertaken essentially as previously described by Sanchez-Corral *et al.*[25] using normal human serum (negative control) and serum from an affected aHUS patient known to carry the CFH/CFHR1 hybrid gene (positive control; Venables *et al.*[43]). Normal human serum was also premixed with increasing quantity of OX-24 (1.2 mg/ml in AP buffer) as needed. See Supplementary Methods online for full details.

(b) *Decay acceleration on sheep erythrocytes*: C3b-coated sheep erythrocytes were prepared as described previously.[24] Cells were resuspended to 2% (vol/vol) in AP buffer. The AP convertase was formed on the cell surface by incubating 50 μl cells with 50 μl of AP buffer containing FB (3 μg/ml) and FD (0.2 μg/ml) at 37 °C for 15 min. The cells (100 μl) were then added to 50 μl of either FH (0.4–50 nmol/l), $FH^{1-5\wedge18-20}$ (0.4–50 nmol/l), or FH^{1-5} (3–400 nmol/l) in PBS/20 mmol/l ethylenediaminetetraacetic acid (EDTA) and incubated at 25 °C for 15 min. Lysis was developed by adding 50 μl of 4% (vol/vol) normal human serum depleted of FB and FH[24] in PBS/20 mM EDTA and incubating at 37 °C for 25 min. To determine the amount of lysis, cells were pelleted by centrifugation, and hemoglobin release was measured at 420 nm (A_{420}). The percentage of inhibition from lysis was calculated by the formula (A_{420} (buffer only) − A_{420} (FH))/A_{420} (buffer only) × 100%. Controls included 0% lysis (buffer only—PBS/20 mM EDTA) and 100% lysis (0.01% Triton in PBS/20 mM EDTA).

(c) *Measuring FH cofactor activity*: C3b-coated sheep erythrocytes were resuspended to 2% (vol/vol) in AP buffer. Fifty microliters of cells was added to an equal volume of AP buffer containing 2.5 μg/ml FI and either FH (0.8–100 nmol/l), $FH^{1-5\wedge18-20}$ (0.8–100 nmol/l), or FH^{1-5} (6–750 nmol/l) and incubated at 25 °C for 8 min. After three washes in AP buffer, cells were resuspended in 50 μl of AP buffer. A further 50 μl of AP buffer containing FB (4 μg/ml) and FD (0.4 μg/ml) was added to cells and incubated at 25 °C for 15 min to form AP convertase on the remaining erythrocyte surface-bound C3b. Lysis was developed by adding 50 μl 4% (vol/vol) normal human serum depleted of FB and FH in PBS/20 mM EDTA and incubating at 37 °C for 20 min. Percentage inhibition of lysis was calculated as in part (b).

Administration of serum-derived FH, $FH^{1-5\wedge18-20}$, and FH^{1-5} to *Cfh −/−* mice

All materials administered to animals were subjected to lipopolysaccharide removal,[44] and were confirmed to be lipopolysaccharide free by the method of Moesby *et al.*[45] Based on the FH dose used successfully in Fakhouri *et al.*,[14] mice were injected intraperitoneally with serum-derived full-length human FH (3 nmol/465 μg per animal), $FH^{1-5\wedge18-20}$ (12 nmol/710 μg), or FH^{1-5} (12 nmol/424 μg) in identical volumes of PBS, or PBS alone. Blood was collected onto EDTA via tail venesection before injection and at serial time points thereafter, with plasma separated via centrifugation for storage at −80 °C. Mice were killed at the indicated time points, and the kidneys were collected into PBS and snap frozen in OCT embedding matrix (CellPath, Newtown, UK) for storage at −80 °C.

Measurement of mouse plasma C3 or FH reagents by ELISA

Mouse C3 levels were measured by ELISA using HRP–conjugated goat polyclonal anti-mouse C3 Ab (MP Biomedicals, Santa Ana, CA; catalog no. 0855557) as described previously.[12] Alternatively, to detect human FH or our constructs, OX-24 (5 μg/ml) was used as capture and goat anti-human FH, followed by bovine anti-goat-HRP used as detection in standard ELISA (see Supplementary Methods online).

Immunostaining of mouse renal sections for murine C3 and C3d, and human FH

Five micrometer cryosections from mouse kidneys were mounted on SuperFrost glass slides (VWR, Lutterworth, UK) coated with 0.1% (v/v) poly-L-lysine in H_2O (Sigma), before fixing in acetone and storage at −80 °C. Thirty-minute blocking and 1 h staining steps were performed at room temperature, with 60 μl applied per thawed section. For mouse C3, blocking was with 20% (v/v) goat serum (Sigma) in PBS, and detection was with 1 in 200 FITC–conjugated goat polyclonal anti-mouse C3 Ab (MP Biomedicals; catalog no. 0855500; 4 mg/ml) in PBS. For mouse C3d, blocking was with 2% BSA/PBS, followed by the Biotin Blocking System (Dako, Glostrup, Denmark), and detection was with 1 in 10 biotinylated polyclonal goat anti-mouse C3d Ab (R&D Systems, Abingdon, UK; catalog no. BAF2655; 50 mg/ml) in 2% BSA/PBS, followed by rinsing and secondary detection with 1 in 400 phycoerythrin-conjugated streptavidin (BD Pharmingen, Oxford, UK; catalogue no. 554061; 0.5 mg/ml) in PBS. For human FH, blocking was with the Biotin Blocking System and detection was with 1 in 25 biotinylated Ox-24 (1 mg/ml) in PBS, followed by rinsing and secondary detection using 1 in 400 phycoerythrin-conjugated streptavidin (BD Pharmingen; catalogue no. 554061; 0.5 mg/ml) in PBS. After rinsing, slides were mounted in VECTASHIELD HardSet Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Burlingame,

CA) and covered with glass coverslips. Sections were visualized at original magnification ×40 using an Olympus BX40 microscope with a BX-FLA vertical fluorescence illuminator (Olympus, Tokyo, Japan). Ten glomeruli per section were identified based on glomerular clusters of DAPI–positive cells. Immunofluorescence images were then captured and quantitative immunofluorescence was performed using a QImaging Retiga 2000R digital camera (QImaging, Surrey, BC, Canada) and Image-Pro Plus 7.0 software (Media Cybernetics, Warrendale, PA). Mean fluorescence intensity for each section is expressed in arbitrary fluorescence units.

Statistical analysis

Data were analyzed using GraphPad Prism 6.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). Groups were compared at each time point using one- or two-way analysis of variance with Bonferroni multiple comparisons test as indicated.

DISCLOSURE

During the completion of the study, E-MN (nee Hunze) has taken up a position with GSK, Stevenage, UK. MCP has received funding from Alexion Pharmaceuticals for preclinical studies of TT30 in *Cfh* – / – mice (manuscript under revision) and lecture fees. All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. (**A**) FH^{1-5} mediated accelerated decay of the AP C3 convertase was measured by surface Plasmon resonance essentially as described in Schmidt *et al*, 2011.
Figure S2. (**A**) Fluid-phase FI cofactor activity of $FH^{1-5\wedge18-20}$ was measured by incubation of C3b and FI with for 30 min at 37°C.
Figure S3. Effect of repeat administration of $FH^{1-5\wedge18-20}$ on glomerular staining of 8 week old, sex matched, $FH^{-/-}$ mice over 24 hours.
Figure S4. Six C57Bl/6 (wild type) mice were injected I.P. with a 3nmol dose of $FH^{1-5\wedge18-20}$ while two mice received only PBS as control.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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